FINAL REPORT

Study Title

Bacterial Reverse Mutation Assay

Testing Guidelines

OECD Guideline 471 (updated and adopted 21 July 1997)

Test Substance

JA900-DAA

Author

Emily W. Dakoulas, B.S.

Study Completion Date

15 June 2015

Testing Facility

BioReliance Corporation
9630 Medical Center Drive
Rockville, MD 20850

BioReliance Study Number

AE19WX.503.BTL

Sponsor

International Flavors & Fragrances Inc.
800 Rose Lane
Union Beach, NJ 07735

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STATEMENT OF COMPLIANCE

Study No. AE19WX.503.BTL was conducted in compliance with the following regulations: US EPA GLP Standards 40 CFR 792 (TSCA) with the following exceptions:

1. The identity, strength, purity, stability and composition or other characteristics to define the test substance were determined by the Sponsor. However, the characterization documents do not indicate the regulations under which the analyses were conducted.

   Study Director Impact Statement: The impact cannot be determined because the appropriate information was not provided to the Study Director. The study conclusion was based on the test substance as supplied.

2. Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

   Study Director Impact Statement: The impact cannot be determined because the appropriate analyses were not performed. The study conclusion was based on the nominal dose levels as documented in the study records.

Emily W. Dakoulas, B.S.
Study Director

15 JUN 2015
Date

QUALITY ASSURANCE STATEMENT

BioReliance by SAFC

Quality Assurance Statement

Study Information

Number: AE19WX.503.BTL

Compliance

Procedures, documentation, equipment and other records were examined in order to assure this study was performed in accordance with the regulation(s) listed below and conducted according to the protocol and relevant Standard Operating Procedures. Verification of the study protocol was performed and documented by Quality Assurance.

US EPA Good Laboratory Standards 40CFR 792

Inspections

Quality Assurance performed the inspections(s) below for this study.

Insp. Dates (From/To)		Phase Inspected	To Study Director	To Management
21-Apr-2015	21-Apr-2015	Protocol Review	21-Apr-2015	21-Apr-2015
22-Apr-2015	22-Apr-2015	Dilution of the test article and/or positive control	22-Apr-2015	22-Apr-2015
15-May-2015	18-May-2015	Data/Draft Report	18-May-2015	18-May-2015
15-Jun-2015	15-Jun-2015	Final Report	15-Jun-2015	15-Jun-2015

The Final Report for this study describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

For a multisite study, test site QA Statements are located in the corresponding contributing scientist report.

E-signature

Quality Assurance: Ellen Burns 15-Jun-2015 5:30 pm GMT

Reason for signature: QA Approval

Printed by:Ellen Burns

Printed on:15-Jun-15

STUDY INFORMATION

Study Conduct

Sponsor:	International Flavors & Fragrances Inc. 800 Rose Lane Union Beach, NJ 07735
Sponsor's Authorized Representative:	Xiao Huang, Ph.D.
Testing Facility:	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
BioReliance Study No.:	AE19WX.503.BTL

Test Substance

Identification:	JA900-DAA (The test substance is a polymer, 51% in ethanol.)
Synonym:	Jeffamine ED900 diacrylamide
Lot No.:	RDLV28986
Purity:	51.4% (per Certificate of Analysis)
Average Molecular Weight (M_n):	Approximately 1000 g/mol (provided by the Sponsor in the protocol) (Actual M_n = 1082, per Certificate of Analysis)
Description:	Clear colorless liquid
Storage Conditions:	Room temperature, protected from light
Receipt Date:	24 March 2015

Study Dates

Study Initiation Date:	10 April 2015
Experimental Start Date:	14 April 2015
Experimental Completion Date:	27 April 2015

Key Personnel

Study Director:	Emily W. Dakoulas, B.S.

Testing Facility Management:	Rohan Kulkarni, MSc, Ph.D. Director, Genetic Toxicology Study Management
Laboratory Supervisor:	Jessica Heavin, M.S.
Report Writer:	Melissa R. VanDyke, B.S.

SUMMARY

The test substance, JA900-DAA, was tested in the Bacterial Reverse Mutation Assay using *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* tester strain WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9. The assay was performed in two phases, using the plate incorporation method. The first phase, the initial toxicity-mutation assay, was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. The second phase, the confirmatory mutagenicity assay, was used to evaluate and confirm the mutagenic potential of the test substance. Dose formulations were adjusted to compensate for the purity (51.4%) of the test substance, using a correction factor of 1.95.

Water was selected as the solvent of choice based on information provided by the Sponsor, the solubility of the test substance and compatibility with the target cells. The formulation prepared for use in the solubility test also was adjusted for test article purity using a correction factor of 1.95. The test substance formed a clear solution in sterile water at a concentration of approximately 50 mg/mL, the maximum concentration tested in the solubility test conducted at BioReliance.

In the initial toxicity-mutation assay, the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 50 mg/mL and a 100 µL plating aliquot. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Neither precipitate nor toxicity was observed. Based on the findings of the initial toxicity-mutation assay, the maximum dose plated in the confirmatory mutagenicity assay was 5000 µg per plate.

In the confirmatory mutagenicity assay, no positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. The dose levels tested were 50, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed.

Under the conditions of this study, JA900-DAA was concluded to be negative in the Bacterial Reverse Mutation Assay.

PURPOSE

The purpose of this study was to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* strain WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. Historical control data are found in Appendix I. A copy of the study protocol is included in Appendix II.

This assay design was based on the testing guidelines of the OECD (1997).

CHARACTERIZATION OF TEST AND CONTROL SUBSTANCES

The test substance was received by BioReliance on 24 March 2015 and was assigned the code number AE19WX. Upon receipt, the test substance was described as a clear, colorless liquid and was stored at room temperature, protected from light.

The Sponsor has determined the identity, strength, purity and composition or other characteristics to define the test substance and the stability of the test substance. A copy of the Certificate of Analysis is included in Appendix III. Based on the expiration date provided in the Certificate of Analysis, the test substance is considered stable through March 2017.

The vehicle used to deliver JA900-DAA to the test system was sterile water.

Vehicle	CAS Number	Supplier	Lot Number	Purity	Expiration Date
Sterile water	7732-18-5	Sigma-Aldrich	RNBD4869	Sterile-filtered	October 2016
			RNBD2494		May 2016

Dose formulations were adjusted to compensate for the purity (51.4%) of the test substance, using a correction factor of 1.95. Test substance dilutions were prepared immediately before use and delivered to the test system at room temperature under filtered light.

Positive controls plated concurrently with each assay are listed in the following table. All positive controls were diluted in dimethyl sulfoxide (DMSO) except for sodium azide, which was diluted in sterile water. All subdivided solutions of positive controls were stored at -10 to -30°C.

Strain	S9 Activation	Positive Control	Concentration (µg/plate)
TA98, TA1535	Rat	2-aminoanthracene (Sigma Aldrich Chemical Co., Inc.) Lot No. STBD3302V Exp. Date 31-Jul-2017 CAS No. 613-13-8 Purity 97.5%	1.0
TA100, TA1537			2.0
WP2 *uvr*A			15
TA98	None	2-nitrofluorene (Sigma Aldrich Chemical Co., Inc.) Lot No. S43858V Exp. Date 31-Mar-2016 CAS No. 607-57-8 Purity 99.4%	1.0
TA100, TA1535		sodium azide (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBH5113V Exp. Date 30-Jun-2016 CAS No. 26628-22-8 Purity 99.6%	1.0
TA1537		9-aminoacridine (Sigma Aldrich Chemical Co., Inc.) Lot No. 09820CEV Exp. Date 31-Mar-2016 CAS No. 52417-22-8 Purity 99.4%	75
WP2 *uvr*A		methyl methanesulfonate (Sigma Aldrich Chemical Co., Inc.) Lot No. MKBR6050V Exp. Date 31-Oct-2017 CAS No. 66-27-3 Purity 100.0%	1,000

The negative and positive control substances have been characterized as per the Certificates of Analysis on file with the testing facility. The stability of the negative and positive control substances and their mixtures was demonstrated by acceptable results that met the criteria for a valid test.

Dose Formulation Collection and Analysis

Analyses to determine the concentration, uniformity and stability of the test substance dose formulations were not performed.

MATERIALS AND METHODS

Test System

The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al*. (1975) and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel (1976). *Salmonella* tester strains were derived from Dr. Bruce Ames' cultures; *E. coli* tester strains were from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

Tester strains TA98 and TA1537 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. Tester strain TA1535 is reverted by mutagens that cause basepair substitutions. Tester strain TA100 is reverted by mutagens that cause both frameshift and basepair substitution mutations. Specificity of the reversion mechanism in *E. coli* is sensitive to basepair substitution mutations, rather than frameshift mutations (Green and Muriel, 1976).

Solubility Test

A solubility test was conducted using sterile water to determine the highest soluble or workable stock concentration up to 50 mg/mL.

Preparation of Tester Strain

Overnight cultures were prepared by inoculating from the appropriate frozen permanent stock into a vessel, containing 30 to 50 mL of culture medium. To assure that cultures were harvested in late log phase, the length of incubation was controlled and monitored. Following inoculation, each flask was placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C for approximately 12 hours before the anticipated time of harvest. Each culture was monitored spectrophotometrically for turbidity and was harvested at a percent transmittance yielding a titer of greater than or equal to 0.3×10^9 cells per milliliter. The actual titers were determined by viable count assays on nutrient agar plates.

Identification of Test System

Each plate was identified by the BioReliance study number and a code system to designate the treatment condition, dose level and test phase, as described in detail in BioReliance's Standard Operating Procedures.

Metabolic Activation System

Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats that were injected intraperitoneally with Aroclor™ 1254 (200 mg/mL in corn oil) at a dose of 500 mg/kg, five days before sacrifice. The S9 (Lot No. 3409, Exp. Date: 04 February 2017) was purchased commercially from MolTox (Boone, NC). Upon arrival at BioReliance, the S9 was stored at -60°C or colder until used. Each bulk

preparation of S9 was assayed for its ability to metabolize benzo(a)pyrene and 2-aminoanthracene to forms mutagenic to *Salmonella typhimurium* TA100.

The S9 mix was prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

The Sham mixture (Sham mix), containing 100 mM phosphate buffer at pH 7.4, was also prepared on the day of use.

Frequency and Route of Administration

The test system was exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983).

Initial Toxicity-Mutation Assay to Select Dose Levels

The initial toxicity-mutation assay was used to establish the dose-range for the confirmatory mutagenicity assay and to provide a preliminary mutagenicity evaluation. Vehicle control, positive controls and eight dose levels of the test substance were plated, two plates per dose, with overnight cultures of TA98, TA100, TA1535, TA1537 and WP2 *uvr*A on selective minimal agar in the presence and absence of Aroclor-induced rat liver S9.

Confirmatory Mutagenicity Assay

The confirmatory mutagenicity assay was used to evaluate and confirm the mutagenic potential of the test substance. Five dose levels of test substance along with appropriate vehicle control and positive controls were plated with overnight cultures of TA98, TA100, TA1535, TA1537 and WP2 *uvr*A on selective minimal agar in the presence and absence of Aroclor-induced rat liver S9. All dose levels of test substance, vehicle control and positive controls were plated in triplicate.

Treatment of Test System

On the day of its use, minimal top agar, containing 0.8 % agar (W/V) and 0.5 % NaCl (W/V), was melted and supplemented with L-histidine, D-biotin and L-tryptophan solution to a final concentration of 50 µM each. Top agar not used with S9 or Sham mix was supplemented with 25 mL of sterile water for each 100 mL of minimal top agar. Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) containing 1.5 % (W/V) agar. Nutrient bottom agar was Vogel-Bonner minimal medium E containing 1.5 % (W/V) agar and supplemented

with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder). Nutrient Broth was Vogel-Bonner salt solution supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder).

To confirm the sterility of the S9 and Sham mixes, a 0.5 mL aliquot of each was plated on selective agar. To confirm the sterility of the test substance and the vehicle, all test substance dose levels and the vehicle used in each assay were plated on selective agar with an aliquot volume equal to that used in the assay. These plates were incubated under the same conditions as the assay.

One-half (0.5) milliliter of S9 or Sham mix, 100 µL of tester strain (cells seeded) and 100 µL of vehicle or test substance dilution were added to 2.0 mL of molten selective top agar at 45±2°C. When plating the positive controls, the test substance aliquot was replaced by a 50 µL aliquot of appropriate positive control. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. After the overlay had solidified, the plates were inverted and incubated for 48 to 72 hours at 37±2°C. Plates that were not counted immediately following the incubation period were stored at 2-8°C until colony counting could be conducted.

Scoring

The condition of the bacterial background lawn was evaluated for evidence of test substance toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification. Toxicity and degree of precipitation were scored relative to the vehicle control plate using the codes shown in the following table. As appropriate, colonies were enumerated either by hand or by machine.

Code	Description	Characteristics
1 or no code	Normal	Distinguished by a healthy microcolony lawn.
2	Slightly Reduced	Distinguished by a noticeable thinning of the microcolony lawn and possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over greater than or equal to 90% of the plate.
6	Obscured by Particulate	The background bacterial lawn cannot be accurately evaluated due to microscopic test substance particulate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than or equal to 10% of the revertant colony count (e.g., less than or equal to 3 particles on a plate with 30 revertants).
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., greater than 3 particles on a plate with 30 revertants). These plates are counted manually.

Tester Strain Verification

On the day of use in each assay, all tester strain cultures were checked for the appropriate genetic markers.

Criteria for a Valid Test

The following criteria must be met for each assay to be considered valid. All *Salmonella* tester strain cultures must demonstrate the presence of the deep rough mutation (*rfa*) and the deletion in the *uvr*B gene. Cultures of tester strains TA98 and TA100 must demonstrate the presence of the pKM101 plasmid R-factor. All WP2 *uvr*A cultures must demonstrate the deletion in the *uvr*A gene. All cultures must demonstrate the characteristic mean number of spontaneous revertants in the vehicle controls as follows (inclusive): TA98, 10 - 50; TA100, 80 - 240; TA1535, 5 - 45; TA1537, 3 - 21; WP2 *uvr*A, 10 - 60. To ensure that appropriate numbers of bacteria are plated, tester strain culture titers must be greater than or equal to 0.3×10^9 cells/mL. The mean of each positive control must exhibit at least a 3.0-fold increase in the number of revertants over the mean value of the respective vehicle control. A minimum of three non-toxic

dose levels is required to evaluate assay data. A dose level is considered toxic if one or both of the following criteria are met: (1) A >50 % reduction in the mean number of revertants per plate as compared to the mean vehicle control value. This reduction must be accompanied by an abrupt dose-dependent drop in the revertant count. (2) At least a moderate reduction in the background lawn (background code 3, 4 or 5).

Evaluation of Test Results

For each replicate plating, the mean and standard deviation of the number of revertants per plate were calculated and are reported.

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0-times the mean vehicle control value.

Strains TA98, TA100 and WP2 *uvr*A

Data sets were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0-times the mean vehicle control value.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response was evaluated as negative if it was neither positive nor equivocal.

Electronic Data Collection Systems

The primary computer or electronic systems used for the collection of data or analysis included, but were not limited to, the following:

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel 2007 (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

Records and Archives

All raw data, the protocol, pertinent study email correspondence and all reports for procedures performed at BioReliance will be maintained in the archives at BioReliance, Rockville, MD for at least five years, unless otherwise requested by the Sponsor. At that time, the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials will first be copied and the copy will be retained by the BioReliance archives in accordance with the applicable SOPs. The raw data, reports and other documents generated at locations other than BioReliance will be archived by the test site. All unused test substance was disposed prior to report finalization.

Deviations

No deviations from the protocol or assay-method SOPs occurred during the conduct of this study

RESULTS AND DISCUSSION

Solubility Test

Water was selected as the solvent of choice based on information provided by the Sponsor, the solubility of the test substance and compatibility with the target cells. The formulation prepared for use in the solubility test also was adjusted for test article purity using a correction factor of 1.95. The test substance formed a clear solution in sterile water at a concentration of approximately 50 mg/mL, the maximum concentration tested in the solubility test conducted at BioReliance.

Sterility Results

No contaminant colonies were observed on the sterility plates for the vehicle control, the test substance dilutions or the S9 and Sham mixes.

Tester Strain Titer Results

Experiment	Tester Strain				
	TA98	TA100	TA1535	TA1537	WP2 *uvr*A
	Titer Value (x 10^9 cells per mL)				
B1	1.9	0.9	1.3	1.3	2.4
B2	3.6	3.6	5.7	7.3	11.6

Initial Toxicity-Mutation Assay

The results of the initial toxicity-mutation assay are presented in Tables 1 and 2. These data were generated in Experiment B1.

In Experiment B1 (Initial Toxicity-Mutation Assay), the maximum dose tested was 5000 µg per plate; this dose was achieved using a concentration of 50 mg/mL and a 100 µL plating aliquot. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg per plate. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. Neither precipitate nor toxicity was observed. Based on the findings of the initial toxicity-mutation assay, the maximum dose plated in the confirmatory mutagenicity assay was 5000 µg per plate.

Confirmatory Mutagenicity Assay

The results of the confirmatory mutagenicity assay are presented in Tables 3 and 4. These data were generated in Experiment B2.

In Experiment B2 (Confirmatory Mutagenicity Assay), no positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation. The

dose levels tested were 50, 150, 500, 1500 and 5000 µg per plate. Neither precipitate nor toxicity was observed.

A copy of the Common Technical Document Tables is included in Appendix IV.

CONCLUSION

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, JA900-DAA did not cause a positive mutagenic response with any of the tester strains in either the presence or absence of Aroclor-induced rat liver S9.

REFERENCES

Ames, B.N., J. McCann and E. Yamasaki (1975) Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian Microsome Mutagenicity Test, Mutation Research, 31:347-364.

Green, M.H.L. and W.J. Muriel (1976) Mutagen testing using trp+ reversion in *Escherichia coli*, Mutation Research 38:3-32.

Maron, D.M. and B.N. Ames (1983) Revised Methods for the *Salmonella* Mutagenicity Test, Mutation Research, 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

Vogel, H.J. and D.M. Bonner (1956) Acetylornithinase of *E. coli*: Partial Purification and Some Properties, J. Biol. Chem., 218:97-106.

TABLE 1
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AE19WX.503.BTL
Study Code: AE19WX
Experiment: B1
Date Plated: 4/14/2015
Exposure Method: Plate incorporation assay
Evaluation Period: 4/17/2015

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**JA900-DAA**	5000 µg	*13*	*0*	*1.0*	13^{M}, 13^{M}
		1500 µg	*13*	*1*	*1.0*	12^{M}, 13^{M}
		500 µg	*13*	*1*	*1.0*	12^{M}, 14^{M}
		150 µg	*19*	*4*	*1.5*	21^{M}, 16^{M}
		50 µg	*11*	*0*	*0.8*	11^{M}, 11^{M}
		15 µg	*16*	*6*	*1.2*	20^{M}, 11^{M}
		5.0 µg	*11*	*1*	*0.8*	12^{M}, 10^{M}
		1.5 µg	*13*	*3*	*1.0*	11^{M}, 15^{M}
	Water	100 µL	*13*	*1*		13^{M}, 12^{M}
TA100	**JA900-DAA**	5000 µg	*107*	*17*	*1.0*	119^{A}, 95^{A}
		1500 µg	*115*	*17*	*1.1*	127^{A}, 103^{A}
		500 µg	*117*	*8*	*1.1*	123^{A}, 111^{A}
		150 µg	*107*	*8*	*1.0*	112^{A}, 101^{A}
		50 µg	*107*	*6*	*1.0*	111^{A}, 103^{A}
		15 µg	*115*	*13*	*1.1*	124^{A}, 105^{A}
		5.0 µg	*119*	*2*	*1.1*	117^{A}, 120^{A}
		1.5 µg	*115*	*11*	*1.1*	107^{A}, 122^{A}
	Water	100 µL	*104*	*6*		108^{A}, 100^{A}
TA1535	**JA900-DAA**	5000 µg	*15*	*1*	*0.7*	16^{A}, 14^{A}
		1500 µg	*26*	*7*	*1.1*	21^{A}, 31^{A}
		500 µg	*27*	*11*	*1.2*	19^{A}, 35^{A}
		150 µg	*21*	*3*	*0.9*	19^{A}, 23^{A}
		50 µg	*19*	*3*	*0.8*	21^{A}, 17^{A}
		15 µg	*23*	*1*	*1.0*	22^{A}, 24^{A}
		5.0 µg	*21*	*5*	*0.9*	24^{A}, 17^{A}
		1.5 µg	*18*	*1*	*0.8*	19^{A}, 17^{A}
	Water	100 µL	*23*	*1*		24^{A}, 22^{A}
TA1537	**JA900-DAA**	5000 µg	*11*	*1*	*1.2*	11^{A}, 10^{A}
		1500 µg	*9*	*2*	*1.0*	7^{A}, 10^{A}
		500 µg	*6*	*5*	*0.7*	2^{A}, 9^{A}
		150 µg	*9*	*2*	*1.0*	7^{A}, 10^{A}
		50 µg	*7*	*1*	*0.8*	6^{A}, 8^{A}
		15 µg	*11*	*4*	*1.2*	14^{A}, 8^{A}
		5.0 µg	*5*	*4*	*0.6*	7^{A}, 2^{A}
		1.5 µg	*5*	*0*	*0.6*	5^{A}, 5^{A}
	Water	100 µL	*9*	*4*		11^{A}, 6^{A}

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

TABLE 1 CONT.
Initial Toxicity-Mutation Assay without S9 activation

Study Number: AE19WX.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AE19WX
Date Plated: 4/14/2015
Evaluation Period: 4/17/2015

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2*uvr*A	**JA900-DAA**	5000 µg	*28*	*3*	*0.8*	30^{A}, 26^{A}
		1500 µg	*25*	*1*	*0.7*	26^{A}, 24^{A}
		500 µg	*28*	*3*	*0.8*	30^{A}, 26^{A}
		150 µg	*37*	*1*	*1.1*	36^{A}, 38^{A}
		50 µg	*35*	*5*	*1.0*	31^{A}, 38^{A}
		15 µg	*28*	*8*	*0.8*	22^{A}, 34^{A}
		5.0 µg	*25*	*1*	*0.7*	26^{A}, 24^{A}
		1.5 µg	*26*	*4*	*0.8*	23^{A}, 29^{A}
	Water	100 µL	*34*	*1*		35^{A}, 33^{A}
TA98	**2NF**	1.0 µg	*126*	*16*	*9.7*	137^{A}, 115^{A}
TA100	**SA**	1.0 µg	*807*	*13*	*7.8*	816^{A}, 797^{A}
TA1535	**SA**	1.0 µg	*716*	*58*	*31.1*	675^{A}, 757^{A}
TA1537	**9AAD**	75 µg	*432*	*184*	*48.0*	562^{A}, 302^{A}
WP2*uvr*A	**MMS**	1000 µg	*256*	*16*	*7.5*	267^{A}, 245^{A}

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

TABLE 2
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AE19WX.503.BTL
Study Code: AE19WX
Experiment: B1
Date Plated: 4/14/2015
Exposure Method: Plate incorporation assay
Evaluation Period: 4/17/2015

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**JA900-DAA**	5000 µg	*24*	*1*	*0.9*	23^{M}, 24^{M}
		1500 µg	*30*	*3*	*1.1*	28^{M}, 32^{M}
		500 µg	*35*	*8*	*1.3*	29^{M}, 40^{M}
		150 µg	*37*	*7*	*1.4*	42^{M}, 32^{M}
		50 µg	*32*	*9*	*1.2*	38^{M}, 25^{M}
		15 µg	*34*	*4*	*1.3*	37^{M}, 31^{M}
		5.0 µg	*29*	*0*	*1.1*	29^{M}, 29^{M}
		1.5 µg	*28*	*8*	*1.0*	33^{M}, 22^{M}
	Water	100 µL	*27*	*1*		28^{M}, 26^{M}
TA100	**JA900-DAA**	5000 µg	*142*	*2*	*1.1*	140^{A}, 143^{A}
		1500 µg	*126*	*8*	*1.0*	131^{A}, 120^{A}
		500 µg	*120*	*16*	*0.9*	131^{A}, 108^{A}
		150 µg	*120*	*16*	*0.9*	108^{A}, 131^{A}
		50 µg	*132*	*6*	*1.0*	136^{A}, 127^{A}
		15 µg	*121*	*17*	*0.9*	133^{A}, 109^{A}
		5.0 µg	*146*	*1*	*1.1*	145^{A}, 146^{A}
		1.5 µg	*131*	*8*	*1.0*	125^{A}, 137^{A}
	Water	100 µL	*129*	*10*		122^{A}, 136^{A}
TA1535	**JA900-DAA**	5000 µg	*16*	*8*	*0.9*	21^{A}, 10^{A}
		1500 µg	*15*	*3*	*0.8*	17^{A}, 13^{A}
		500 µg	*18*	*2*	*1.0*	16^{A}, 19^{A}
		150 µg	*21*	*6*	*1.2*	25^{A}, 17^{A}
		50 µg	*18*	*2*	*1.0*	16^{A}, 19^{A}
		15 µg	*17*	*1*	*0.9*	18^{A}, 16^{A}
		5.0 µg	*15*	*10*	*0.8*	22^{A}, 8^{A}
		1.5 µg	*13*	*0*	*0.7*	13^{A}, 13^{A}
	Water	100 µL	*18*	*7*		23^{A}, 13^{A}
TA1537	**JA900-DAA**	5000 µg	*16*	*3*	*1.5*	18^{A}, 14^{A}
		1500 µg	*14*	*1*	*1.3*	13^{A}, 15^{A}
		500 µg	*13*	*4*	*1.2*	10^{A}, 15^{A}
		150 µg	*16*	*1*	*1.5*	16^{A}, 15^{A}
		50 µg	*8*	*0*	*0.7*	8^{A}, 8^{A}
		15 µg	*14*	*5*	*1.3*	10^{A}, 17^{A}
		5.0 µg	*11*	*3*	*1.0*	9^{A}, 13^{A}
		1.5 µg	*10*	*1*	*0.9*	10^{A}, 9^{A}
	Water	100 µL	*11*	*5*		7^{A}, 14^{A}

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

TABLE 2 CONT.
Initial Toxicity-Mutation Assay with S9 activation

Study Number: AE19WX.503.BTL
Experiment: B1
Exposure Method: Plate incorporation assay

Study Code: AE19WX
Date Plated: 4/14/2015
Evaluation Period: 4/17/2015

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
WP2*uvr*A	**JA900-DAA**	5000 µg	*34*	*2*	*1.2*	35^{A}, 32^{A}
		1500 µg	*25*	*1*	*0.9*	24^{A}, 26^{A}
		500 µg	*45*	*8*	*1.6*	51^{A}, 39^{A}
		150 µg	*32*	*10*	*1.1*	39^{A}, 25^{A}
		50 µg	*40*	*1*	*1.4*	40^{A}, 39^{A}
		15 µg	*27*	*11*	*1.0*	19^{A}, 34^{A}
		5.0 µg	*29*	*4*	*1.0*	32^{A}, 26^{A}
		1.5 µg	*31*	*11*	*1.1*	39^{A}, 23^{A}
	Water	100 µL	*28*	*8*		22^{A}, 33^{A}
TA98	**2AA**	1.0 µg	*422*	*47*	*15.6*	389^{A}, 455^{A}
TA100	**2AA**	2.0 µg	*725*	*14*	*5.6*	735^{A}, 715^{A}
TA1535	**2AA**	1.0 µg	*125*	*7*	*6.9*	120^{A}, 130^{A}
TA1537	**2AA**	2.0 µg	*57*	*12*	*5.2*	48^{A}, 65^{A}
WP2*uvr*A	**2AA**	15 µg	*378*	*19*	*13.5*	391^{A}, 364^{A}

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

TABLE 3
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AE19WX.503.BTL
Study Code: AE19WX
Experiment: B2
Date Plated: 4/22/2015
Exposure Method: Plate incorporation assay
Evaluation Period: 4/27/2015

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**JA900-DAA**	5000 µg	*28*	*9*	*1.5*	39^{A}, 23^{A}, 23^{A}
		1500 µg	*20*	*9*	*1.1*	17^{A}, 13^{A}, 30^{A}
		500 µg	*27*	*17*	*1.4*	45^{A}, 25^{A}, 11^{A}
		150 µg	*16*	*3*	*0.8*	20^{A}, 14^{A}, 14^{A}
		50 µg	*22*	*2*	*1.2*	23^{A}, 20^{A}, 22^{A}
	Water	100 µL	*19*	*1*		18^{A}, 20^{A}, 18^{A}
TA100	**JA900-DAA**	5000 µg	*99*	*16*	*1.1*	104^{A}, 82^{A}, 112^{A}
		1500 µg	*81*	*18*	*0.9*	92^{A}, 60^{A}, 92^{A}
		500 µg	*94*	*11*	*1.0*	98^{A}, 103^{A}, 82^{A}
		150 µg	*77*	*3*	*0.8*	78^{A}, 80^{A}, 74^{A}
		50 µg	*83*	*30*	*0.9*	62^{A}, 117^{A}, 69^{A}
	Water	100 µL	*93*	*14*		85^{A}, 109^{A}, 86^{A}
TA1535	**JA900-DAA**	5000 µg	*8*	*2*	*1.0*	8^{A}, 10^{A}, 7^{A}
		1500 µg	*13*	*1*	*1.6*	12^{A}, 13^{A}, 13^{A}
		500 µg	*10*	*1*	*1.3*	11^{A}, 10^{A}, 10^{A}
		150 µg	*10*	*3*	*1.3*	13^{A}, 8^{A}, 10^{A}
		50 µg	*9*	*3*	*1.1*	12^{A}, 6^{A}, 10^{A}
	Water	100 µL	*8*	*3*		6^{A}, 12^{A}, 6^{A}
TA1537	**JA900-DAA**	5000 µg	*7*	*1*	*1.2*	8^{A}, 6^{A}, 7^{A}
		1500 µg	*7*	*3*	*1.2*	10^{A}, 7^{A}, 5^{A}
		500 µg	*7*	*4*	*1.2*	5^{A}, 12^{A}, 5^{A}
		150 µg	*5*	*1*	*0.8*	5^{A}, 6^{A}, 5^{A}
		50 µg	*6*	*1*	*1.0*	5^{A}, 7^{A}, 6^{A}
	Water	100 µL	*6*	*1*		6^{A}, 6^{A}, 7^{A}
WP2*uvr*A	**JA900-DAA**	5000 µg	*27*	*2*	*1.0*	27^{A}, 26^{A}, 29^{A}
		1500 µg	*29*	*2*	*1.0*	27^{A}, 30^{A}, 29^{A}
		500 µg	*25*	*10*	*0.9*	31^{A}, 13^{A}, 31^{A}
		150 µg	*31*	*5*	*1.1*	32^{A}, 35^{A}, 26^{A}
		50 µg	*24*	*0*	*0.9*	24^{A}, 24^{A}, 24^{A}
	Water	100 µL	*28*	*6*		35^{A}, 25^{A}, 25^{A}

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

TABLE 3 CONT.
Confirmatory Mutagenicity Assay without S9 activation

Study Number: AE19WX.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AE19WX
Date Plated: 4/22/2015
Evaluation Period: 4/27/2015

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**2NF**	1.0 µg	*107*	*14*	*5.6*	104^{A}, 122^{A}, 94^{A}
TA100	**SA**	1.0 µg	*671*	*95*	*7.2*	781^{A}, 607^{A}, 626^{A}
TA1535	**SA**	1.0 µg	*488*	*40*	*61.0*	490^{A}, 447^{A}, 526^{A}
TA1537	**9AAD**	75 µg	*105*	*1*	*17.5*	104^{A}, 104^{A}, 106^{A}
WP2*uvr*A	**MMS**	1000 µg	*221*	*31*	*7.9*	251^{A}, 222^{A}, 189^{A}

Key to Positive Controls

2NF	2-nitrofluorene
SA	sodium azide
9AAD	9-Aminoacridine
MMS	methyl methanesulfonate

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

TABLE 4
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AE19WX.503.BTL
Study Code: AE19WX
Experiment: B2
Date Plated: 4/22/2015
Exposure Method: Plate incorporation assay
Evaluation Period: 4/27/2015

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**JA900-DAA**	5000 µg	*31*	*5*	*1.5*	26^{A}, 32^{A}, 36^{A}
		1500 µg	*30*	*7*	*1.4*	22^{A}, 32^{A}, 36^{A}
		500 µg	*24*	*4*	*1.1*	19^{A}, 27^{A}, 25^{A}
		150 µg	*29*	*9*	*1.4*	32^{A}, 19^{A}, 35^{A}
		50 µg	*23*	*4*	*1.1*	20^{A}, 23^{A}, 27^{A}
	Water	100 µL	*21*	*2*		24^{A}, 20^{A}, 20^{A}
TA100	**JA900-DAA**	5000 µg	*100*	*10*	*1.2*	97^{A}, 92^{A}, 112^{A}
		1500 µg	*93*	*11*	*1.1*	100^{A}, 80^{A}, 99^{A}
		500 µg	*107*	*2*	*1.3*	108^{A}, 108^{A}, 104^{A}
		150 µg	*102*	*28*	*1.2*	93^{A}, 133^{A}, 80^{A}
		50 µg	*103*	*22*	*1.3*	79^{A}, 106^{A}, 123^{A}
	Water	100 µL	*82*	*1*		81^{A}, 82^{A}, 82^{A}
TA1535	**JA900-DAA**	5000 µg	*12*	*5*	*1.5*	14^{A}, 16^{A}, 7^{A}
		1500 µg	*11*	*3*	*1.4*	14^{A}, 12^{A}, 8^{A}
		500 µg	*14*	*2*	*1.8*	13^{A}, 12^{A}, 16^{A}
		150 µg	*11*	*3*	*1.4*	11^{A}, 8^{A}, 13^{A}
		50 µg	*9*	*2*	*1.1*	7^{A}, 11^{A}, 10^{A}
	Water	100 µL	*8*	*4*		5^{A}, 12^{A}, 6^{A}
TA1537	**JA900-DAA**	5000 µg	*7*	*1*	*0.8*	7^{A}, 8^{A}, 6^{A}
		1500 µg	*11*	*4*	*1.2*	14^{A}, 6^{A}, 12^{A}
		500 µg	*11*	*3*	*1.2*	8^{A}, 11^{A}, 14^{A}
		150 µg	*11*	*3*	*1.2*	12^{A}, 8^{A}, 14^{A}
		50 µg	*11*	*2*	*1.2*	13^{A}, 11^{A}, 10^{A}
	Water	100 µL	*9*	*3*		5^{A}, 11^{A}, 11^{A}
WP2*uvr*A	**JA900-DAA**	5000 µg	*34*	*6*	*1.2*	30^{A}, 32^{A}, 41^{A}
		1500 µg	*25*	*9*	*0.9*	22^{A}, 19^{A}, 35^{A}
		500 µg	*30*	*6*	*1.0*	36^{A}, 27^{A}, 26^{A}
		150 µg	*32*	*1*	*1.1*	33^{A}, 32^{A}, 31^{A}
		50 µg	*26*	*11*	*0.9*	14^{A}, 36^{A}, 27^{A}
	Water	100 µL	*29*	*6*		36^{A}, 26^{A}, 25^{A}

Key to Automatic & Manual Count Flags

^{M}: Manual count ^{A}: Automatic count

TABLE 4 CONT.
Confirmatory Mutagenicity Assay with S9 activation

Study Number: AE19WX.503.BTL
Experiment: B2
Exposure Method: Plate incorporation assay

Study Code: AE19WX
Date Plated: 4/22/2015
Evaluation Period: 4/27/2015

Strain	Substance	Dose level per plate	Mean revertants per plate	Standard Deviation	Ratio treated / solvent	Individual revertant colony counts and background codes
TA98	**2AA**	1.0 µg	*176*	*57*	*8.4*	239[A], 160[A], 129[A]
TA100	**2AA**	2.0 µg	*523*	*80*	*6.4*	615[A], 487[A], 467[A]
TA1535	**2AA**	1.0 µg	*128*	*7*	*16.0*	125[A], 123[A], 136[A]
TA1537	**2AA**	2.0 µg	*96*	*31*	*10.7*	88[A], 70[A], 131[A]
WP2*uvr*A	**2AA**	15 µg	*358*	*164*	*12.3*	534[A], 331[A], 210[A]

Key to Positive Controls

2AA 2-aminoanthracene

Key to Automatic & Manual Count Flags

[M]: Manual count [A]: Automatic count

APPENDIX I: Historical Control Data

Historical Negative and Positive Control Values
2014

Revertants per plate

Strain	Control	Activation									
		None					Rat Liver				
		Mean	SD	Min	Max	95% CL	Mean	SD	Min	Max	95% CL
TA98	Neg	16	5	5	42	6-26	24	7	5	53	10-38
	Pos	232	258	57	2691		400	165	109	1382	
TA100	Neg	94	14	66	152	66-122	102	18	63	164	66-138
	Pos	681	176	213	1767		681	259	186	2793	
TA1535	Neg	11	4	2	31	3-19	13	5	2	36	3-23
	Pos	586	226	16	2509		117	99	23	1060	
TA1537	Neg	7	3	1	19	1-13	9	4	1	23	1-17
	Pos	411	355	32	2921		72	52	10	562	
WP2 *uvr*A	Neg	25	7	7	62	11-39	28	8	10	55	12-44
	Pos	376	123	99	1026		302	102	91	687	

SD=standard deviation; Min=minimum value; Max=maximum value; 95% CL = Mean ±2 SD (but not less than zero); Neg=negative control (including but not limited to deionized water, dimethyl sulfoxide, ethanol and acetone); Pos=positive control

APPENDIX II: Study Protocol

BioReliance by SAFC

Protocol

Study Title	**Bacterial Reverse Mutation Assay**
Study Director	Emily Dakoulas, BS
Testing Facility	BioReliance Corporation 9630 Medical Center Drive Rockville, MD 20850
Sponsor	International Flavors & Fragrances Inc. 800 Rose Lane Union Beach, NJ 07735
Sponsor's Authorized Representative	Xiao Huang, PhD
BioReliance Study Number	AE19WX.503.BTL

1. KEY PERSONNEL

Study Director	Emily Dakoulas, BS BioReliance Corporation Phone: 301-610-2153 Email: emily.dakoulas@bioreliance.com
BioReliance Quality Assurance Representative	Karen Westray, RQAP-GLP BioReliance Corporation Phone: 301-610-2856 Email: karen.westray@bioreliance.com
Sponsor's Authorized Representative	Xiao Huang, PhD International Flavors & Fragrances Inc. 800 Rose Lane Union Beach, NJ 07735 Phone: 732-203-8136 Fax: 732-203-8176 Email: xiao.huang@iff.com

2. TEST SCHEDULE

Proposed Experimental Initiation Date	14 April 2015
Proposed Experimental Completion Date	06 May 2015
Proposed Report Date	20 May 2015

3. REGULATORY REQUIREMENTS

This study will be performed in compliance with the following Good Laboratory Practices (GLP) regulations.

- US EPA GLP Standards 40 CFR 792 (TSCA)

At a minimum, all work performed at US test site(s) will comply with the US GLP regulations stated above. Non-US sites must follow the GLP regulations governing their site. The regulations that were followed will be indicated on the compliance statement in the final contributing report.

4. QUALITY ASSURANCE

The protocol, any amendments, at least one in-lab phase, the raw data, draft report(s), and final report(s) will be audited by BioReliance Quality Assurance (QA) and a signed QA Statement will be included in the final report.

Test Site Quality Assurance (where applicable)

Test Site QA is responsible for performing an in-lab phase inspection, auditing raw data and final report(s), and providing the inspection results to the Principal Investigator, Study Director, and their respective management. A signed QA Statement documenting the type of audit performed, the dates it was performed, and the dates in which the audit results were reported to the Study Director,

Principal Investigator and their respective management must be submitted by the test site QA.

5. PURPOSE

The purpose of this study is to evaluate the mutagenic potential of the test substance by measuring its ability to induce reverse mutations at selected loci of several strains of *Salmonella typhimurium* and at the tryptophan locus of *Escherichia coli* WP2 *uvr*A in the presence and absence of an exogenous metabolic activation system. The assay design is based on the OECD Guideline 471, updated and adopted 21 July 1997.

6. TEST SUBSTANCE INFORMATION

Identification	JA900-DAA*
Synonyms	Jeffamine ED900 diacrylamide*
	(*The test substance is a polymer, 51% in ethanol)
Storage Conditions	Room Temperature Protect from light
Purity	51.4% (a correction factor of 1.95 will be used for dose formulations)
Average Molecular Weight (M_n)	Approximately 1000 g/mol

Characterization of Test Substance
Characterization of the Test Substance is the responsibility of the Sponsor.

Test Substance Reserve Sample
A reserve sample of the Test Substance is the responsibility of the Sponsor.

Characterization of Dose Formulations
Dose formulations will not be analyzed.

Stability of Test Substance in Vehicle
Stability of Test Substance in Vehicle, under the conditions of use, is the responsibility of the Sponsor.

Disposition of Test Substance and Dose Formulations
All unused test substance will be disposed prior to report finalization unless the test substance is used on another study. Residual dose formulations will be discarded after use.

7. TEST SYSTEM

The tester strains will include the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 as described by Ames *et al.* (1975) and the *E. coli* tester strain WP2 *uvr*A as described by Green and Muriel (1976). The genotypes of strains are as follows:

Histidine Mutation			Tryptophan Mutation	Additional Mutations		
*his*G46	*his*C3076	*his*D3052	*trp*E	LPS	Repair	R-factor
TA1535	TA1537	-	-	*rfa*	Δ*uvr*B	-
TA100	-	TA98	-	*rfa*	Δ*uvr*B	+R
-	-	-	WP2 *uvr*A	-	Δ*uvr*A	-

The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). The tester strains may also be obtained from Molecular Toxicology Inc. (Moltox).

8. EXPERIMENTAL DESIGN AND METHODOLOGY

The test system will be exposed to the test substance via the plate incorporation methodology originally described by Ames *et al.* (1975) and updated by Maron and Ames (1983). This test system has been shown to detect a wide range of classes of chemical mutagens (McCann *et al.*, 1975; McCann and Ames, 1976).

If the Sponsor is aware of specific metabolic requirements (e.g., azo compounds), this information will be utilized in designing the assay.

Solubility Determination

According to the sponsor, the test substance is soluble in water.

As needed, a solubility determination will be conducted to determine the maximum soluble concentration or workable suspension as indicated below. Vehicles compatible with this test system, in order of preference, include but are not limited to deionized water (CAS 7732-18-5), dimethyl sulfoxide (CAS 67-68-5), ethanol (CAS 64-17-5) and acetone (CAS 67-64-1). The vehicle of choice, selected in order of preference, will be that which permits preparation of the highest workable or soluble stock concentration, up to 50 mg/mL for aqueous vehicles and up to 500 mg/mL for organic vehicles. Based on the molecular weight of the test substance, the vehicles to be tested and the dose to be achieved in the assay, alternate stock concentrations may be tested, as needed.

Preparation of Tester Strain

Each tester strain culture will be inoculated from the appropriate frozen stock, lyophilized pellet(s), or master plate. To ensure that cultures are harvested in late log phase, the length of incubation will be controlled and monitored. Each inoculated

flask will be placed in a shaker/incubator programmed to begin shaking at 125 to 175 rpm and incubating at 37±2°C.

All cultures will be harvested by spectrophotometric monitoring of culture turbidity rather than by duration of incubation since overgrowth of cultures can cause loss of sensitivity to some mutagens. Cultures will be removed from incubation at a density of approximately 10^9 cells/mL.

Identification of Test System
Each plate will be identified by the BioReliance study number and a code system to designate at least the treatment condition, dose level, and test phase.

Exogenous Metabolic Activation

Liver Homogenate
Liver homogenate (S9) will be purchased commercially (MolTox; Boone, NC). It is prepared from male Sprague-Dawley rats that have been injected intraperitonealy with Aroclor™ 1254 (200 mg/mL in corn oil), at a dose of 500 mg/kg, 5 days before sacrifice.

Sham Mix
100 mM phosphate buffer at pH 7.4

S9 Mix
S9 mix will be prepared on the day of use as indicated below:

Component	Final Concentration
β-nicotinamide-adenine dinucleotide phosphate	4 mM
Glucose-6-phosphate	5 mM
Potassium chloride	33 mM
Magnesium chloride	8 mM
Phosphate Buffer (pH 7.4)	100 mM
S9 homogenate	10% (v/v)

Controls
No analyses will be performed on the positive control substances or the positive control dose formulations. The neat positive control substances and the vehicles used to prepare the test substance and positive control formulations will be characterized by the Certificates of Analysis provided by the Supplier(s). Copies of the Certificates of Analysis will be kept on file at BioReliance.

Vehicle Control
The vehicle for the test substance will be used as the vehicle control for each treatment group. For vehicles with no historical control data, an untreated control will be included.

Sterility Controls
At a minimum, the most concentrated test substance dilution and the Sham and S9 mixes will be checked for sterility.

Positive Controls
Results obtained from these substances will be used to assure responsiveness of the test system but not to provide a standard for comparison with the test substance.

Strain	Positive Control	S9	Concentrations (µg/plate)
Salmonella strains	2-aminoanthracene[B]	+	1.0 – 2.0
WP2 *uvr*A	2-aminoanthracene[B]	+	10 – 20
TA98	2-nitrofluorene[B]	–	1.0
TA100, TA1535	sodium azide[A]	–	1.0
TA1537	9-aminoacridine[B]	–	75
WP2 *uvr*A	methyl methanesulfonate[B]	–	1,000

[A]Prepared in water
[B]Prepared in DMSO

Frequency and Route of Administration
The test system will be treated using the plate incorporation method.

Verification of a clear positive response will not be required (OECD Guideline 471). Equivocal results will be retested in consultation with the Sponsor using an appropriate modification of the experimental design (e.g., dose levels, activation system or treatment method).

Initial Toxicity-Mutation Assay to Select Dose Levels
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least eight concentrations of test substance, in duplicate, in both the presence and absence of S9. Unless limited by solubility, the test substance will be evaluated at a maximum concentration of 5000 µg/plate. Unless indicated otherwise by the Sponsor, the dose levels will be 5000, 1500, 500, 150, 50, 15, 5.0 and 1.5 µg/plate. If limited by solubility in the vehicle, the test substance will be evaluated at the highest concentration permissible as a workable suspension. Dose levels for the confirmatory mutagenicity assay will be based upon post-treatment toxicity, the precipitation profile, solubility of the test substance and will be documented in the raw data and report. If the top dose is less than 5000 µg/plate due to precipitation or solubility issues, the Sponsor will be consulted. If a retest of the initial toxicity-mutation assay is needed, a minimum of five dose levels of test substance will be used in the retest.

Confirmatory Mutagenicity Assay
TA98, TA100, TA1535, TA1537 and WP2 *uvr*A will be exposed to vehicle alone and at least five concentrations of test substance, in triplicate, in both the presence and absence of S9.

Treatment of Test System
Unless specified otherwise, test substance dilutions will be prepared immediately prior to use. All test substance dosing will be at room temperature under filtered light. One half milliliter (0.5 mL) of S9 mix or Sham mix, 100 µL of tester strain and 50 µL of vehicle, test substance dilution or positive control will be added to 2.0 mL of molten selective top agar at 45±2°C. When necessary, aliquots of other than 50 µL of test substance or vehicle or positive control will be plated. When plating untreated controls, the addition of test substance, vehicle and positive control will be omitted. The mixture will be vortex mixed and overlaid onto the surface of a minimal bottom agar plate. After the overlay has solidified, the plates will be inverted and incubated for 48 to 72 hours at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring
The condition of the bacterial background lawn will be evaluated for evidence of test substance toxicity and precipitate. Evidence of toxicity will be scored relative to the vehicle control plate and recorded along with the revertant count for that plate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification. As appropriate, colonies will be enumerated either by hand or by machine.

Tester Strain Verification
On the day of use in the initial toxicity-mutation assay and the confirmatory mutagenicity assays, all tester strain cultures will be checked for the appropriate genetic markers.

9. CRITERIA FOR DETERMINATION OF A VALID TEST

The following criteria must be met for the initial toxicity-mutation assay and the confirmatory mutagenicity assay to be considered valid. If one or more of these parameters are not acceptable, the affected condition(s) will be retested.

Tester Strain Integrity
To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvr*B mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the *uvr*A mutation, all *E. coli* tester strain cultures must exhibit sensitivity to ultraviolet light. To demonstrate the presence of the pKM101 plasmid R-factor, tester strain cultures of TA98 and TA100 must exhibit resistance to ampicillin.

Vehicle Controls Values
Based on historical control data, all tester strain cultures must exhibit characteristic numbers of spontaneous revertants per plate in the vehicle controls. The mean revertants per plate must be within the following ranges (inclusive): TA98, 10 - 50;

TA100, 80 - 240; TA1535, 5 - 45; TA1537, 3 - 21; WP2 *uvr*A, 10 - 60. Untreated controls, when part of the design, must also be within the ranges cited above.

Tester Strain Titers
To ensure that appropriate numbers of bacteria are plated, all tester strain culture titers must be equal to or greater than $0.3x10^9$ cells per milliliter.

Positive Control Values
Each mean, positive control value must exhibit at least a 3.0-fold increase over the respective mean, vehicle control value for each tester strain.

Toxicity
A minimum of three non-toxic dose levels will be required to evaluate assay data. A dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean vehicle control value (this reduction must be accompanied by an abrupt dose-dependent drop in the revertant count) or a reduction in the background lawn. In the event that less than three non-toxic dose levels are achieved, the affected portion of the assay will be repeated with an appropriate change in dose levels.

10. EVALUATION OF TEST RESULTS

For the test substance to be evaluated positive, it must cause a dose-related increase in the mean revertants per plate of at least one tester strain over a minimum of two increasing concentrations of test substance as specified below:

Strains TA1535 and TA1537
Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 3.0-times the mean vehicle control value.

Strains TA98, TA100 and WP2 *uvr*A
Data sets will be judged positive if the increase in mean revertants at the peak of the dose response is equal to or greater than 2.0-times the mean vehicle control value.

An equivocal response is a biologically relevant increase in a revertant count that partially meets the criteria for evaluation as positive. This could be a dose-responsive increase that does not achieve the respective threshold cited above or a non-dose responsive increase that is equal to or greater than the respective threshold cited. A response will be evaluated as negative, if it is neither positive nor equivocal.

11. ELECTRONIC DATA COLLECTION SYSTEMS

Electronic systems used for the collection or analysis of data may include but not be limited to the following (version numbers are maintained in the system documentation):

System	Purpose
LIMS Labware System	Test Substance Tracking
Excel (Microsoft Corporation)	Calculations
Sorcerer Colony Counter and Ames Study Manager (Perceptive Instruments)	Data Collection/Table Creation
Kaye Lab Watch Monitoring system (Kaye GE)	Environmental Monitoring
BRIQS	Deviation and audit reporting

12. REPORT

A report of the results of this study will accurately describe all methods used for generation and analysis of the data. The report will include, but not limited to information about the following:

- Test substance
- Vehicle
- Strains
- Test conditions
- Results
- Discussion of results
- Conclusion
- Appendices: Historical Control Data (vehicle and positive controls with ranges, means and standard deviations), copy of protocol and any amendment, contributing reports (if applicable), and, if provided by the Sponsor, copies of the analyses that characterized the test substance, its stability and the stability and strength of the dosing preparations.
- Statement of Compliance
- Quality Assurance Statement
- CTD Tables (unless otherwise requested)

The report will be issued as a QA-audited draft. After receipt of the Sponsor's comments a final report will be issued. A GLP Compliance Statement signed by the Study Director will also be included in the final report and will note any exceptions if the characterization of the test substance and/or the characterization of the dose formulations are not performed or provided. Four months after issuance of the draft report, if no communication regarding the study is received from the Sponsor or designated representative, the draft report may be issued as a final report. If all supporting documents have not been provided, the report will be written based on those that are provided.

13. RECORDS AND ARCHIVES

All raw data, the protocol, pertinent study email correspondence, and all reports for procedures performed at BioReliance will be maintained in the archives at BioReliance, Rockville, MD for at least five years, unless otherwise requested by the Sponsor. At that time, the Sponsor will be contacted for a decision as to the final disposition of the materials. All study materials will first be copied and the copy will be retained by the BioReliance archives in accordance with the applicable SOPs. Slides and/or specimens (as applicable) will be archived at EPL Archives and indexed as such in the BioReliance archive database. The raw data, reports, and other

documents generated at locations other than BioReliance will be archived by the test site.

14. REFERENCES

Ames, B.N., McCann, J. and Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. Mutation Research 31:347-364.

Green, M.H.L., and Muriel, W.J. (1976). Mutagen testing using trp^+ reversion in *Escherichia coli*. Mutation Research 38:3-32.

McCann, J. and Ames, B.N. (1976). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals: discussion. Proc. Natl. Acad. Sci. USA 73:950-954.

McCann, J., Choi, E., Yamasaki, E. and Ames, B.N. (1975). Detection of carcinogens as mutagens in the *Salmonella*/microsome test: assay of 300 chemicals. Proc. Natl. Acad. Sci. USA 72:5135-5139.

Maron, D.M. and Ames, B.N. (1983). Revised Methods for the *Salmonella* Mutagenicity Test. Mutation Research 113:173-215.

OECD Guideline 471 (Genetic Toxicology: Bacterial Reverse Mutation Test), Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, adopted July 21, 1997.

APPROVALS

Sponsor Approval

XIAO HUANG 04/03/2015

Sponsor Representative Date

Study Director and Test Facility Management Approvals

BioReliance Study Director — Date: 10APR2015

BioReliance Study Management — Date: 10Apr2015

APPENDIX III: Certificate of Analysis

INTERNATIONAL FLAVORS & FRAGRANCES (IFF R&D) 1515 HIGHWAY 36, UNION BEACH, NJ 07735 (732) 264-4500
CREATORS AND MANUFACTURERS OF FLAVORS, FRAGRANCES AND AROMA CHEMICALS CABLE: INTERIFF NEW YORK

Certificate of Analysis

JA900-DAA; Jeffamine ED900 diacrylamide; lot RDLV28986.

JA900-DAA, lot RDLV28986, is 51.4% pure, has M_n = 1082 and meets all analytical standards set by International Flavors & Fragrances, Inc. The expiration date is 3/2017.

This material should be stored ambient, protected from light.

Lisa Veliath, PhD
Research Investigator
International Flavors & Fragrances
1515 Highway 36
Union Beach, NJ 07735
732-335-2871

March 20, 2015

APPENDIX IV: Common Technical Document Tables

Report Title: Bacterial Reverse Mutation Assay
Test for Induction of: Reverse mutation in bacterial cells
Species/Strain: *S. typhimurium, E. coli*

No. of Independent Assays: 2
No. of Replicate Cultures: 2 (#1) and 3 (#2)

Test Substance: JA900-DAA
Study No.: AE19WX.503.BTL
No. Cells Analyzed/Culture: 0.9 to 11.6 x 10^8 cells per plate
GLP Compliance: Yes

Metabolizing System: Aroclor-induced rat liver S9
Vehicle for Test Substance: Sterile water
Vehicle for Positive Controls: DMSO, except sterile water for sodium azide
Treatment: Plate incorporation
Date(s) of Treatment: 14 April 2015 (#1) and 22 April 2015 (#2)
Cytotoxic Effects: None
Genotoxic Effects: None

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Initial Toxicity-Mutation Assay (#1) Revertant Colony Counts (Mean ±SD)				
			TA98	TA100	TA1535	TA1537	WP*2uvr*A
Without Activation	Water	100 µL/plate	13 ± 1	104 ± 6	23 ± 1	9 ± 4	34 ± 1
	JA900-DAA	1.5	13 ± 3	115 ± 11	18 ± 1	5 ± 0	26 ± 4
		5.0	11 ± 1	119 ± 2	21 ± 5	5 ± 4	25 ± 1
		15	16 ± 6	115 ± 13	23 ± 1	11 ± 4	28 ± 8
		50	11 ± 0	107 ± 6	19 ± 3	7 ± 1	35 ± 5
		150	19 ± 4	107 ± 8	21 ± 3	9 ± 2	37 ± 1
		500	13 ± 1	117 ± 8	27 ± 11	6 ± 5	28 ± 3
		1500	13 ± 1	115 ± 17	26 ± 7	9 ± 2	25 ± 1
		5000	13 ± 0	107 ± 17	15 ± 1	11 ± 1	28 ± 3
	2NF	1.0	126 ± 16				
	SA	1.0		807 ± 13	716 ± 58		
	9AAD	75				432 ± 184	
	MMS	1000					256 ± 16

Key to Positive Controls

SA	sodium azide
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Initial Toxicity-Mutation Assay (#1) Revertant Colony Counts (Mean ±SD)				
			TA98	TA100	TA1535	TA1537	WP*2uvr*A
With Activation	Water	100 µL/plate	27 ± 1	129 ± 10	18 ± 7	11 ± 5	28 ± 8
	JA900-DAA	1.5	28 ± 8	131 ± 8	13 ± 0	10 ± 1	31 ± 11
		5.0	29 ± 0	146 ± 1	15 ± 10	11 ± 3	29 ± 4
		15	34 ± 4	121 ± 17	17 ± 1	14 ± 5	27 ± 11
		50	32 ± 9	132 ± 6	18 ± 2	8 ± 0	40 ± 1
		150	37 ± 7	120 ± 16	21 ± 6	16 ± 1	32 ± 10
		500	35 ± 8	120 ± 16	18 ± 2	13 ± 4	45 ± 8
		1500	30 ± 3	126 ± 8	15 ± 3	14 ± 1	25 ± 1
		5000	24 ± 1	142 ± 2	16 ± 8	16 ± 3	34 ± 2
	2AA	1.0	422 ± 47		125 ± 7		
	2AA	2.0		725 ± 14		57 ± 12	
	2AA	15					378 ± 19

Key to Positive Controls

2AA 2-aminoanthracene

Metabolic Activation	Test Substance	Dose Level (µg/plate)	Confirmatory Mutagenicity Assay (#2) Revertant Colony Counts (Mean ±SD)				
			TA98	TA100	TA1535	TA1537	WP2*uvr*A
Without Activation	Water	100 µL/plate	19 ± 1	93 ± 14	8 ± 3	6 ± 1	28 ± 6
	JA900-DAA	50	22 ± 2	83 ± 30	9 ± 3	6 ± 1	24 ± 0
		150	16 ± 3	77 ± 3	10 ± 3	5 ± 1	31 ± 5
		500	27 ± 17	94 ± 11	10 ± 1	7 ± 4	25 ± 10
		1500	20 ± 9	81 ± 18	13 ± 1	7 ± 3	29 ± 2
		5000	28 ± 9	99 ± 16	8 ± 2	7 ± 1	27 ± 2
	2NF	1.0	107 ± 14				
	SA	1.0		671 ± 95	488 ± 40		
	9AAD	75				105 ± 1	
	MMS	1000					221 ± 31
With Activation	Water	100 µL/plate	21 ± 2	82 ± 1	8 ± 4	9 ± 3	29 ± 6
	JA900-DAA	50	23 ± 4	103 ± 22	9 ± 2	11 ± 2	26 ± 11
		150	29 ± 9	102 ± 28	11 ± 3	11 ± 3	32 ± 1
		500	24 ± 4	107 ± 2	14 ± 2	11 ± 3	30 ± 6
		1500	30 ± 7	93 ± 11	11 ± 3	11 ± 4	25 ± 9
		5000	31 ± 5	100 ± 10	12 ± 5	7 ± 1	34 ± 6
	2AA	1.0	176 ± 57		128 ± 7		
	2AA	2.0		523 ± 80		96 ± 31	
	2AA	15					358 ± 164

Key to Positive Controls

SA	sodium azide
2AA	2-aminoanthracene
9AAD	9-Aminoacridine
2NF	2-nitrofluorene
MMS	methyl methanesulfonate